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In vitro expression and mutagenesis of a gene for corticotropin releasing factor

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ABSTRACT

The specific goals of this proposal are to: (1) create a recombinant gene for corticotropin releasing factor (**CRF**), (2) express that gene by in vitro transcription and translation, (3) test the function of this recombinant protein by receptor binding assay and agonist-induced release of ACTH from cutured pituitary cells and (4) create and test mutants of the CRF molecule (starting at the level of the DNA). We have accomplished the first two of these goals and partially completed the third. We have synthesized the CRF gene, expressed it and characterized the recombinant protein. This protein is active when applied to pituitary cells, but the in vitro translation extract contains substances which partially interfere with that activity. We are presently purifying the recombinant protein from the translation extract.

In a related area, we are conducting experiments to characterize the stress non-responsive period (**SNRP**) in the neonatal rat. We find that the spontaneously hypertensive rat (**SHR**) is not entirely subject to this quiescent adrenocortical period (during the first two weeks of neonatal life) when compared with the normotensive control animal. This difference is not caused by alterations in the levels of circulating (or stored) ACTH, implying that there are differences in the responsiveness of the adrenal cortex.

Further characterization of the SNRP indicates that cultured pituitary corticotroph cells display differing responses to exogenous CRF depending on the age of the animal. At early ages (1 and 6 days postnatally), the cells produce reduced maximal release of ACTH during increasing CRF stimulation. They are more sensitive, however, to CRF stimulation - that is, they will respond to 100-fold lower levels of CRF.

INTRODUCTION

The hypothalamus-pituitary-adrenocortical axis is the primary mediator of the mammalian endocrine response to stress. Stressful stimula trigger neuronal activity in the hypothalamus which results in the release of the hormone corticotropin releasing factor (CRF) into the hypophysial portal system. Blood carries the CRF to specific cells (corticotrophs) in the anterior pituitary which respond by releasing a hormone of their own, adrenocorticotropin (ACTH), into the systemic circulation. Carried to the adrenal cortex, ACTH elicits the liberation of the glucocorticoids. These are the primary agents in many of an organism's physiological reactions to stress. This scheme is grossly simplified in that there are several other transmitter/hormone systems which are known to regulate steroid metabolism and release. However, it is generally accepted that CRF is the primary regulatory element in the stress-induced release of ACTH from the pituitary

(rev. in 1 and 2). This is most dramatically demonstrated in experiments of Rivier and co-workers where injection of rabbit antisera (against CRF), into rats, was shown to block stress-induced increases in both ACTH and corticosterone (3).

CRF has recently been purified from a number of sources and has been characterized as a 41 amino acid peptide (4-8). The peptide shows marked conservation of amino acid sequence across species boundaries. In particular, the rat and human hormones are identical. Perhaps more striking, CRF from quite disparate sources demonstrates remarkable functional homology. Specifically, the ovine CRF (oCRF), which varies from the rat/human CRF (r/hCRF) by seven amino acids, is fully capable of binding to rat pituitary receptors in a manner indistinguishable from the rat hormone itself. This latter finding has greatly facilitated *in vivo* and *in vitro* characterization of the CRF molecule.

The genes for oCRF and hCRF have been cloned and sequenced (9,10). Sequence analysis of an oCRF cDNA clone indicates that the precursor for the hormone is a peptide of 190 amino acids. The prohormone contains a signal sequence at its amino terminal which is typical of most peptide hormones (11). This is then followed by sequences which bear striking homology to the arginine vasopressin-neurophysin II precursor and to the ACTH- -Lipotropin precursor. It has been suggested, based on these observations, that these three hormones are evolutionarily related (9). The carboxyl end of the oCRF prohormone contains the sequences representing the hormone. In fact, during processing, only the final two amino acids of the prohormone are removed to liberate the intact carboxyl terminus. The prohormone is proteolytically processed following translation at recognition sites typical of other peptide hormones. That is, the amino terminus of the mature hormone sequence is preceded, in the prohormone, by the amino acid sequence arg-lys-arg-arg. This amino acid array appears to be a standard peptide processing signal (12). During the removal of the carboxyl terminal amino acids from the hormone, the C-terminus is amidated (13). Analysis of a human genomic clone indicates that the gene is relatively simple and small, being contained within 2000 bp of DNA (10). Moreover, the transcription unit of the gene is interrupted by a single intron (800 bp) present in the 5' untranslated region.

Relatively little work has addressed the structure/function relationships within the CRF molecule - that is, which amino acids are critical for the binding of the peptide to its receptor and the subsequent stimulation of ACTH release. A report by Rivier *et al.* (14) indicates that removal of five or six amino acids from the amino terminus produces peptides with full agonist activity but reduced potency. A deletion of seven amino acids, however, produces an antagonist - a compound which binds to the receptor but does

not elicit ACTH release. At the carboxyl terminus, removal of a single amino acid (ala-41 in oCRF) produces a full agonist - albeit a weak one. These workers also synthesized a mutant peptide designed to maximize the alpha-helical nature of the central part of the CRF molecule. This reconstructed peptide has been shown to be two to three times more potent than wild-type oCRF as an agonist eliciting ACTH release from rat pituitary cells in vitro. During the course of these studies, it was shown that the injection of a CRF antagonist, into rats, can block the stress-induced release of ACTH. As a result of this elegant paper, we are constructing a preliminary picture of how CRF interacts with its receptor and elicits ACTH release.

These studies, however, suffer from several limitations. The experiments utilized the ovine CRF sequence which has been shown to differ from the r/hCRF at seven positions (4,10). Although these hormones exhibit identical activities, it is difficult to predict how the amino acid changes will affect the function of partial peptides. This is particularly true at the carboxyl terminus where the removal of a single amino acid (ala in oCRF; ile in r/hCRF) attenuates the agonist potency of oCRF. Second, the studies did not analyze, in depth, the nature of the amino acid sequences involved in receptor recognition of the peptide. The mutational analysis may have been limited by the fact that the peptides were chemically synthesized in a costly and time-consuming manner. For example, the synthesis of a single, 41-amino acid peptide by the solid phase, chemical method would cost a minimum of $2000. We propose to circumvent these problems by utilizing an in vitro expression system to synthesize the peptide hormone from a reconstructed cDNA clone for the rat/human CRF gene. Our previous experience with this system suggests that we will be able to construct a number of mutants of CRF (both deleted and amino acid substituted) at a fraction of the cost and time required to chemically synthesize the protein.

We propose to explore the nature of the interaction of corticotropin releasing factor (CRF) with its cognate receptor in the pituitary gland. Our intent is to address both the binding characteristics of CRF mutants as well as their ability to elicit the release of ACTH from pituitary cells. We will pursue this goal using a recently described and novel technique of molecular biology to synthesize CRF (both wild-type and mutated forms) in vitro from a reconstructed gene for the protein.

In a another series of experiments, we are examining the hormonal status of neonatal rats in an effort to establish mechanisms which are involved in two separate stress-related phenomena. The first situation involves the early postnatal period in the rat which is characterized by unperturbable, low levels of circulating corticosterone. This time of adrenocortical quiescence is referred to as the stress non-responsive period (SNRP; reviewed in ref. 15). We are interested in investigating the involvement of pituitary corticotroph

responsiveness in this SNRP. The second situation involves the ontogeny of hormone responsiveness in a genetic rat model for essential hypertension.

The mechanisms underlying the developmental elevation of blood pressure in spontaneously hypertensive rats (SHR) are not known. Morphological and physiological abnormalities in the pituitary and adrenal glands of SHR are indicated by a progressive increase in adrenal hyperplasia and an increase in the population of ACTH-producing basophil cells (16,17) as well as an increase in the basal level of circulating corticosterone (18,19). These findings suggest that the hypothalamic-pituitary-adrenocortical (HPA) axis may play a role in the development of hypertension. Significantly higher blood pressures have been observed in SHR compared to WKY animals at early postnatal periods. Differences between the two strains have been documented even on the day of birth. There are extensive cardiovascular alterations which are evident by the weanling stage in SHR. In view of the evident changes which are occurring during the early development (pre-weaning) of the SHR, we have undertaken the preliminary characterization of the HPA axis during this period.

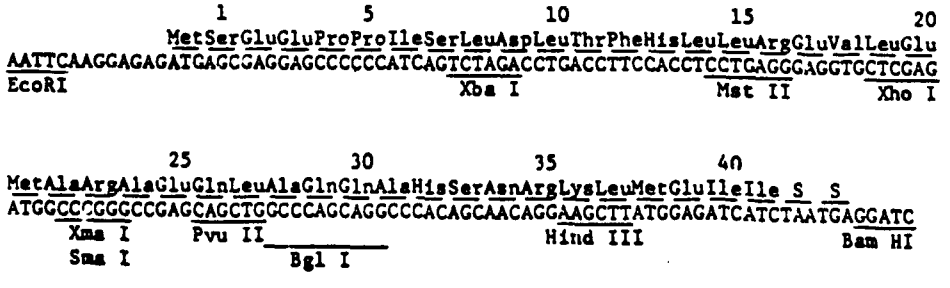
METHODS AND RESULTS

Synthesis of functional proteins following transciption and translation (in vitro) of a full-length cDNA clone

The central goal of the present proposal is to synthesize, in vitro, functional corticotropin releasing factor from its cDNA clone. Briefly, a cDNA clone, carried on an SP6 transcription vector, is transcribed using SP6 RNA polymerase as described by Melton and co-workers (20,21). In order to produce translation-competent mRNA, the 5' end of the message is "capped" by including a 7-methyl guanosine dinucleotide precursor in the transcription reaction. Although the mRNA thus produced is not 100% capped, it will support translation at significant levels. In vitro translation of this synthetic message is accomplished by established procedures (22) using a cell-free wheat germ extract prepared in the laboratory.

Construction of a synthetic corticotropin releasing factor cDNA

In the design of these experiments, we have taken significant liberties with the definition of a "cDNA clone". The diagram below shows a map of the CRF cDNA construct which we have prepared.



Several points should be noted about this construct. It is not a true cDNA clone in that it was not synthesized from mRNA. Rather, this gene was synthesized in toto (relying on published amino acid sequence; ref. 4,10) on a DNA oligonucleotide synthesizer (as three separate fragments). Moreover, the sequence does not resemble, with a high degree of homology, that of the true mRNA. As I discussed earlier, native CRF is synthesized as part of a larger prohormone which is then proteolytically processed to yield mature CRF (9, 10). We have performed that processing at the DNA level when constructing this gene.

The first 13 nucleotides are derived from the 5'-untranslated sequence of the Xenopus TFIIIA gene and were chosen because they are readily utilized by the wheat germ translation extract. Because translation must initiate with a methionine residue, we have included an initiator codon (ATG) prior to amino acid position number 1 (of the CRF gene). Finally, although this DNA sequence codes for the exact amino acid sequence of r/hCRF (plus an initiator methionine), we have altered third position codon nucleotides (with a computer program) to create the constellation of restriction endonuclease sites shown below the nucleotide sequence. This will permit us great latitude in creating deletion and amino acid substitution mutants in this gene. As a consequence of these alterations, however, this construct possesses only 80% sequence homology, at the nucleotide level, with the wild-type gene (10). For that reason, this gene would prove to be a poor hybridization probe for Northern or Southern blots.

Transcription and translation (in vitro) of the cDNA gene for corticotropin releasing factor

After sequencing the construct to confirm its identity, we subcloned the fragment into EcoRI/BamHI-digested pSP6-5 such that transcription of the BamHI-linearized construct using the SP6 promoter will yield sense strand RNA (mRNA). We designate this plasmid pSPCRF-1. The analogous construct, cloned in the opposite orientation with reference to the promoter (in pSP6-4), will produce anti-sense RNA and is designated pSPCRF-2. Transcription of both of these plasmids, following linearization, produces RNA molecules of the predicted size when analyzed on denaturing, Maxam-Gilbert sequencing gels (23).

These capped mRNA molecules have been translated in vitro in a cell-free wheat germ extract. By using translation amino acid mixtures lacking methionine and supplemented with ^{35}S-methionine, we have been able to monitor protein synthesis in these reactions.

A surprise arises when we compare the molecular weight of the putative CRF with ^{14}C-labelled molecular weight markers. Computer analysis of the sequence of CRF predicts a molecular weight of 4800 Da for the peptide. Based on the markers, however, the molecular weight of our synthesized protein is closer to 3000 Da. It is common for small peptides (less that 10 kDa) to migrate anomolously on polyacrylamide gels. We therefore analyzed the translation products in a manner independent of gel mobility. We subjected samples of our in vitro synthesized peptide, commercial CRF and a mixture of the two peptides to SDS-polyacrylamide gel electrophoresis. The gel was stained with Coomassie blue and then subjected to autoradiography. Not only did the unlabelled and synthesized proteins migrate to the same position in the gel, but in the mixture of the two, the bands (radiolabelled and stained) were coincident. We interpret this result to indicate that, in spite of the fact that the peptides do not display the expected molecular weight, our synthesized hormone is indistinguishable from the wild-type following electrophoresis.

We have quantified the yield of the transcription/translation system by excising the protein bands from the above gel and similar experiments. In general, we find that we produce approximately 2 pmol of CRF (10 ng) in a 100 uL reaction. We have confirmed this yield and further established the identity of the recombinant CRF with a radioimmunoassay using CRF-specific polyclonal antiserum.

Characterization of the agonist activity of the recombinant CRF

We have been forced to abandon Specific Aim #2: Demonstrating the ligand binding activity of the recombinant peptide using receptor binding activity with rat pituitary membranes. We have made three concerted efforts to establish this binding assay within the laboratory. These attempts have included sending a student to the laboratory where the assay originated. Even so, we do not obtain satisfactory background signals to permit the use of this procedure in the analysis of our recombinant peptide.

Given our difficulties with the binding assay, we have turned our attention towards establishing the agonist activity of the synthetic CRF by analyzing its ability to elicit the release of ACTH from pituitary cells. We originally had evidence that recombinant CRF possessed agonist activity when applied to cultured AtT-20 cells (a mouse pituitary tumor cell line which secretes ACTH in response to CRF). This was described in the previous progress report. In the months which followed, we encountered difficulty getting reproducible release of ACTH from these cells. We now believe that the problem resided with the tumor cell line and not the synthetic CRF. We have shown this by establishing a system for the primary culture of rat pituitary cells. Pituitaries

are removed from rats and the cells dispersed with collagenase. Following culturing for 3 to 5 days, the cells attach to culture dishes and are triggered to release ACTH by the addition of CRF to the medium. This system has proven to be much more sensitive than the tumor cell line and produces a much higher release of ACTH (3 to 5-fold over unstimulated cells). Using this culture system, we have shown that the synthetic CRF displays agonist activity with an affinity within 5-fold of the wild-type peptide. Unfortunately, we have recently discovered that wheat germ translation extracts which have been prepared in the absence of CRF mRNA (and hence do not contain CRF peptide) also will trigger release of ACTH. This apparent activity is less than the extract containing CRF. We are currently attempting to purify the recombinant CRF from the wheat germ extract to obviate this problematic background stimulation. We are using gel-permeation chromatography and HPLC to effect this purification.

Hormone profiles in the neonatal spontaneously hypertensive rat.

We have completed the first phase of our studies on the ontogeny of the stress response in the neonatal spontaneously hypertensive rat. The results of this study have recently been published (24). Using an ACTH radioimmunoassay and a competitive binding assay for corticosterone, we have measured the circulating plasma levels for these two hormones in SHR and the normotensive Wistar-Kyoto (WKY) strain of rat. We examined these values at two different ages: 10 days postnatally (a period when rats do not respond to stress) and 20 days of age (just prior to weaning and when the rats do respond to stress. In addition, stored levels of these hormones were measured in the pituitary glands of these animals. Circulating corticosterone was significantly lower, in both strains, at 10 days than at 20 days. Although glucocorticoids were undetectable in WKY animals at 10 days, significant levels were observed in age-matched SHR. No difference in corticosterone concentrations was observed between the two strains at 20 days. Circulating ACTH levels did not reflect the values for circulating corticosteroids. Moreover, pituitary stores of ACTH between animals of different strains and ages were not found to be different among any of the groups tested.

These results demonstrate that there is a difference in circulating corticosterone levels between spontaneously hypertensive and Wistar-Kyoto rats at ten days postnatally which is not evident just prior to weaning (20 days). This difference is not due to variations in stored or circulating ACTH. Indeed, ACTH levels are high at a time (10 days) when corticosterone is low - thus suggesting that the difference may reside within the responsiveness of the adrenal cortex. In addition, the spontaneously hypertensive animals are maintaining steroid levels (or responding to stressful stimuli) in the midst of the "stress non-responsive period" (SNRP) at 10 days of age. We are currently pursuing this apparent discrepancey in the responsiveness of the

SHR by increasing the number of data points tested (1, 6, 11, 16, and 20 days of age) while simultaneously examining the levels of a pivotal adrenal medullary enzyme (tyrosine hydroxylase; **TH**). Not only is this enzyme important for the stress response (it is the rate-limiting enzyme in epinephrine biosynthesis), but it is also known to be regulated by glucocorticoids. We will examine both the enzyme activity levels and the mRNA for the enzyme in an effort to establish if there is a functional consequence of the altered steroid levels.

Involvement of pituitary corticotrophs in the SNRP

As an extension of the previously discussed experiments, we are examining the role of the pituitary corticotroph cells in the maintenance of the stress non-responsive period. We are in the midst of examining the ability of primary pituitary cultures to respond to the addition of CRF as a function of the age of the donor animals. We performing this series of experiments in conjunction with our expanded characterization of the SHR model for hypertension. In addition to the WKY normotensive control, we are examining the Sprague-Dawley strain of rat. Preliminary data suggest that at early ages (1 and 6 days), rat pituitary cultures demonstrate a reduced maximal release of ACTH in response to increasing levels of exogenously added CRF. However, they have a dramatically increased sensitivity to CRF - that is, they respond to at least 100-fold lower concentrations of CRF than adult pituitaries. As with the previous experiments, the various strains of rat are not different in terms of their pituitary response or circulating ACTH levels. The differences appear to reside in the adrenal cortex.

CONCLUSIONS AND OUTLOOK

We have completed approximately 50% of the specific aims outlined in the original contract proposal. We have succesfully sythesized a recombinant cDNA clone for CRF and expressed that clone in vitro. We are making slower progress, than anticipated, on the characterization of the recombinant peptide's activity. This can be attributed to our inability to establish a CRF-receptor binding assay and our difficulty establishing an agonist activity assay involving cultured pituitary cells. We believe that we have solved the latter problem through the use of primary rat pituitary cultures. We are now in the process of purifying the synthetic CRF from the wheat germ translation extract in an effort to eliminate problematic substances found in the extract.

We have made great progress in the related area of hypothalamic-pituitary-adrenocortical (HPA) function during the stress non-responsive period (SNRP) in the neonatal rat. We have demonstrated that a rat model for hypertension displays aa alteration in its hormone profile

during this period. Specifically, the spontaneously hypertensive rat possesses circulating glucocorticoids during the SNRP at a time when the normotensive control displays no detectable glucocorticoids. Indeed, the rat does not normally respond to stress during this period by secreting steroids. The difference in the SHR appears to reside in the functioning of the adrenal cortex. We are currently pursuing this problem relative not only to the HPA axis but also relative to the status of the adrenal medulla (by examining the regulation of tyrosine hydroxylase gene expression).

Finally, we are assessing the role of the pituitary corticotroph cell in the maintenance on the neonatal stress non-responsive period. Preliminary findings suggest that cultured pituitary cells are more sensitive to CRF signals early in development. Their innate ability to respond, however, appears to be reduced. We are further examining this phenomenon with the goal of further characterizing the mechanisms underlying the stress response. Moreover, we may be able to integrate these findings with the in vitro expression of recombinant CRF by using the more sensitive neonatal cells to study CRF agonist activity.

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